XF Glycolysis Stress Test Kit

User Manual

For use with the
XF24 Analyzer

Part# 102194-100

For Research Use Only

Preface

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I. Introduction

Overview

Cellular metabolism is the process of converting fuel substrates, such as glucose, fatty acids, and glutamine, to ATP and building blocks, through a series of enzymatic oxidation and reduction reactions. Cells take up reactants from their environment, such as glucose and O_2, and process them biochemically to generate ATP, resulting in the extrusion of products such as lactate, H^+ and CO_2, into the extracellular environment. ATP produced is used to maintain cellular function and to do work.

The Seahorse XF Analyzer, simultaneously measures these energy producing pathways non-invasively, in real-time, through the use of novel, solid-state sensors, specially designed consumables, and elegant instrument mechanics. The rate of oxygen consumption, or OCR, is proportional to mitochondrial respiration, while the rate of extracellular acidification, through the extrusion of protons, is proportional to glycolysis. Changes in O_2 and H^+ concentrations are measured in a transient microchamber, with the solid-state sensors residing 200 microns above the cell surface.

This ability to measure cellular metabolism has enabled scientists worldwide to advance their understanding of the roles glycolysis, and mitochondrial respiration, play in human physiology and disease. Changes in cellular metabolism have been shown to have therapeutic and diagnostic potential in the areas of obesity, diabetes, aging, cancer, cardiovascular function, immunology, and more recently, translational medicine.

Assay Principle

Glycolysis and oxidative phosphorylation are the two major energy producing pathways in the cell. Most cells possess the ability to shift dynamically between these two processes, adapting metabolically to changes in their environment for the purpose of survival. Glucose in the cell is converted to pyruvate, which is then converted to lactate in the cytoplasm, or CO2 and water in the mitochondria. Glucose conversion to lactate (Glycolysis) results in a net production and extrusion of protons into the extracellular medium. As glycolysis occurs, the resulting acidification of the medium surrounding the cells is measured directly by the XF Analyzer and reported as the Extracellular Acidification Rate, or ECAR.

When oxidative phosphorylation is disrupted by an ATP synthase inhibitor (Oligomycin), cells may increase the rate of glycolysis maintain ATP production, and thereby energy homeostasis, as previously shown (Wu M, *et al*.). This elevated rate of glycolysis is referred to as the Glycolytic Capacity of the cell.

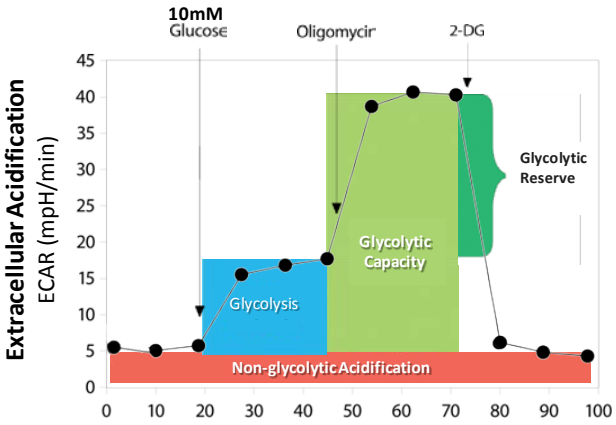


Figure 1. XF Glycolysis Stress Test Profile

This assay provides a standard and comprehensive method to assess the three key parameters of glycolytic function: Glycolysis, Glycolytic Capacity, and Glycolytic Reserve.

ECAR of cells are first assessed in the absence of extracellular glucose to measure non-glycolytic acidification, which results from other metabolic pathways occurring in the cells. Three different compounds, glucose, oligomycin and 2-Deoxy-D-glucose (2-DG) are injected consecutively, followed by 3 ECAR measurements after each injection. The first injection is a saturating concentration of glucose (10 mM). Glucose is taken up by the cells and catabolized through the glycolytic pathway to lactate, producing ATP and protons. The extrusion of protons into the surrounding medium produces a rapid increase in ECAR. This glucose-induced response is reported as the rate of glycolysis (or glycolytic flux) under a basal condition. The second injection is oligomycin, an ATP synthase inhibitor. Oligomycin inhibits mitochondrial ATP production and thus shifts the energy production to glycolysis, with the subsequent increase in ECAR revealing the maximum glycolytic capacity of the cells. The final injection is 2-DG, a glucose analog, which inhibits glycolysis through competitive binding to glucose hexokinase, the first enzyme in the glycolytic pathway. The resulting decrease in ECAR further confirms that the ECAR produced in the experiment is due to glycolysis. The difference between Glycolytic Capacity and Glycolysis rate defines Glycolytic Reserve.

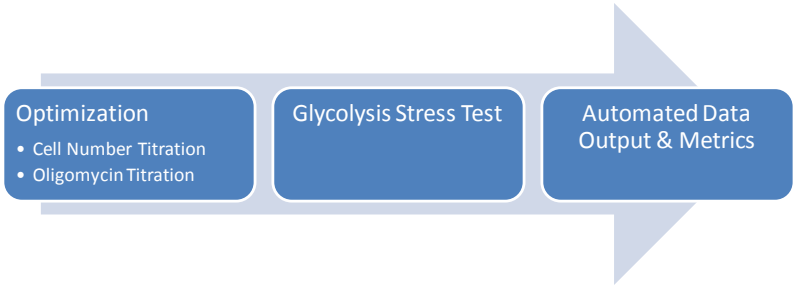


Figure 2. General workflow of the XF Glycolysis Stress Test Kit

XF Glycolysis Stress Test Kit Contents

The XF Glycolysis Stress Test Kit contains enough reagents to run 6 full XF assay plates.

Figure 3. XF Glycolysis Stress Test Kit Contents

Reagent	Quantity
Seahorse Glucose Solution	6 vials of a 2.5 M solution
Seahorse Oligomycin	1 vial of powder + 6 empty vials for reconstituted aliquots
Seahorse 2-DG	1 vial of powder + 6 empty vials for reconstituted aliquots
Seahorse DMSO	1 vial of 1 mL

Materials Required (Not provided in the kit):

- XF24 FluxPak (Seahorse Bioscience Cat. No. 100850-001)
- Serial dilution vessel (24-well plate or 1.5 mL tubes)
- CO_2-free incubator set to 37°C
- XF Glycolysis Stress Test recommended assay medium, as described on page 7.

- Materials for XF Glycolysis Stress Test Media
    - Bicarbonate-free, low phosphate DMEM (Sigma D5030)
    - NaCl
    - Phenol Red (e.g. Sigma P0290)
    - Glucose (e.g. Sigma G8760)
    - L-glutamine (e.g. Invitrogen 25030)

II. Preparing the Components of the Assay

Preparation of Assay Media

There are two types of assay medium utilized in the XF Glycolysis Stress Test. Seahorse recommends preparing the following base medium, formulated for long-term stability, and supplementing with additional components immediately prior to running the assay.

XF Glycolysis Stress Test Base Medium **(Prepare the Day Prior to the Assay)**

Formulation for 1 L:

Component	Concentration
DMEM D 5030 (Sigma)	1 bottle (powder)
NaCl	143 mM (final)
Phenol Red	3 mg

Preparation Instructions:

1. Dissolve one bottle of Sigma D5030 powder in 990 mL of TC-grade water pre-warmed to 37^{o}C
2. Add 1.85 g NaCl for a final concentration of 143 mM
3. Add 0.6 mL of a 0.5% Phenol Red solution (Sigma P0290) for a final concentration of 3 mg/L
4. Adjust the pH to 7.35 +/- 0.05
5. Sterile filter and store at 4^{o}C. It is stable at 4°C for at least 3 months.

XF Glycolysis Stress Test Optimization Medium (Prepare the Day of the Assay)

Preparation of 100 mL (*sufficient for one assay plate)*:

1. Place 100 mL of XF Glycolysis Stress Test Base Medium in an appropriate container
2. Add 400 µl of a 2.5 M Glucose solution for a final concentration of 10 mM
3. Add 1mL of a 200 mM L-glutamine solution for a final concentration of 2 mM
4. Warm to 37 ^{o}C and adjust the pH to 7.35+/- 0.05

XF Glycolysis Stress Test Assay Medium (Prepare the Day of the Assay)

Preparation of 100 mL (*sufficient for one assay plate)*:

1. Place 100 mL of XF Glycolysis Stress Test Base Medium in an appropriate container
2. Add 1mL of a 200 mM L-glutamine solution for a final concentration of 2 mM
3. Warm to 37 ^{o}C and adjust the pH to 7.35+/- 0.05

Reagent Preparation

NOTE: Wear chemically-resistant, impervious gloves when handling the compounds in the kit. Reagent preparation must be performed in a Class II Biological Safety Cabinet. Reagent aliquots must be stored at –20°C. Use reagent aliquots within 3 months of reconstitution.

Remove the XF Glycolysis Stress Test Kit from the freezer and allow the Oligomycin and 2-DG stock vials to warm to room temperature. Store the remaining components in the freezer until the reagent preparations are complete.

Preparation of Oligomycin Aliquots (5 mM)

1. Spin down the stock vial in a micro centrifuge for approximately 5 seconds prior to opening
2. Resuspend powder in 180 µl of DMSO
3. Transfer 30 µl to each aliquot vial. Store vials not needed immediately at -20°C
4. Record the date of resuspension on the side of the XF Glycolysis Stress Test Kit

Preparation of 2-Deoxy-D-Glucose aliquots (1 M)

NOTE: pH adjustment of 2-DG solution may be performed in the reagent bottle or in a separate beaker. Before starting, ensure the availability of an appropriately-sized stir bar.

1. Re-suspend stock in 16 ml of XF Glycolysis Stress Test Assay Medium, pre-warmed to 37°C, pipetting up and down several times
2. Adjust pH to 7.35 +/- 0.05 in stock jar or separate beaker, and adjust volume to 18 ml with XF Glycolysis Stress Test Assay Medium
   NOTE: If solution has cooled to below 30C, it should be warmed in a water bath prior to pH adjustment
3. Transfer 3 ml to each aliquot vial. Store vials not needed immediately at -20°C
4. Record the date of resuspension on the side of the XF Glycolysis Stress Test Kit

Thaw a fresh aliquot of Oligomycin and 2-DG for each assay; warm to room temperature before use.

III. Optimization Assays

Cell Number Titration

Metabolic rates (OCR and ECAR) can vary significantly between different cell types, culturing conditions, and experimental treatments. Therefore, the optimal cell seeding density should be determined on any new cell type before running other optimization or profiling assays.

For the best results, several cell densities should be tested. A typical range is between 10,000 and 100,000 cells per well, although the actual seeding densities may need to be adjusted depending on cell diameter, rate of growth, and other factors. Cell types that are small in diameter, have long doubling times, or which are known to be less metabolically active may require higher seeding densities to achieve optimal results.

An example plate layout diagram is provided below:

X	20k	30k	40k	50k	60k
10k	20k	30k	X	50k	60k
10k	20k	X	40k	50k	60k
10k	20k	30k	40k	50k	X

NOTE: "X" wells should contain only Optimization Medium (no cells).

Prior to the Day of Assay

Cell Seeding

Seed cells ~24 hours prior to the assay in an XF cell culture microplate, and place in a 37°C incubator with the desired level of CO_2.

NOTE: As the actual number of cells present in the well will affect the rate of glycolysis observed, it is strongly recommended to maintain a consistent number of hours between cell seeding and running the XF assay to reduce variation between experiments.

NOTE: For experiments on non-adherent cells, seeding can be performed immediately prior to the experiment. Contact Seahorse Bioscience Support for more details.

Hydrating the XF Sensor Cartridge

The XF Sensor Cartridge must be hydrated prior to the assay:

- Add 1.0 mL of Seahorse Bioscience Calibrant to each well of an XF Utility Plate.
- Place the XF Sensor Cartridge on top of the utility plate, and place in a 37°C incubator **without** CO_2 for a minimum of 12 hours.

Day of Assay

Preparation of XF Glycolysis Stress Test Optimization Medium

Prepare the XF Glycolysis Stress Test Optimization Medium from the Assay Base Medium, as described on page 7.

Perform the Medium Exchange

NOTE: Medium exchange described here is designed to produce a > 2,000 fold dilution of the original culture medium

1. Remove all but 50 µl of the culture medium from each well.
2. Rinse cells two times with 600 µl of XF Stress Test Glycolysis Optimization Medium, pre-warmed to 37°C.
3. Add 750 µl to each well for a final volume of 800 µl/well
4. Place the plate in a 37°C incubator **without** CO_2 for one hour prior to the assay

NOTE: The Seahorse XF Prep Station is recommended to ensure accurate final volumes during media exchanges.

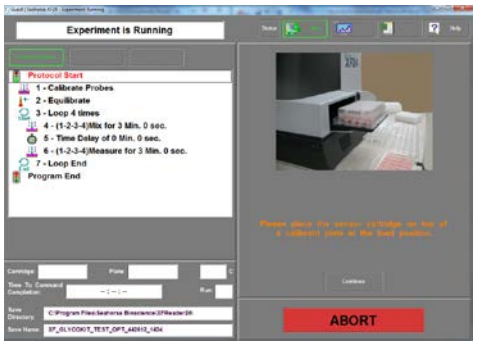
Running the Assay with the XF Glycolysis Stress Test Software App

1. Open the XF software
2. In the Seahorse Apps drop-down menu, choose XF Glycolysis Kit
3. Click the Start App button
4. Click the Run Optimization Plate button
5. Select Cell Number Titration from the drop down menu
6. Enter the Cell Line Name in the appropriate field

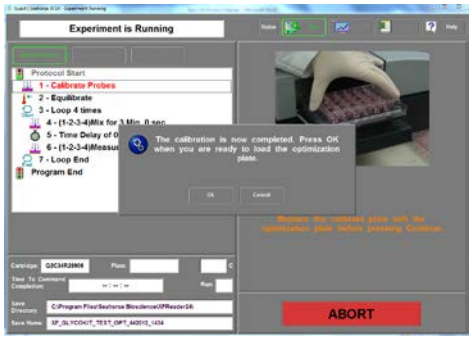
7. Indicate the cell seeding density for each column by clicking on any well in the column, and entering the seeding density used
8. Click the Start button
9. Choose a directory to save the file in, choose a file name, if necessary, and click OK

XF Glycolysis Stress Test
Titration Selection
Cell Number Titration
Cell Line Name:
Enter the cell number titration for each column by clicking on a well.
Start

10. Place the cartridge and calibration plate with loaded injection ports on the sliding tray
11. Click Continue to start calibration


Experiment is Running
Protocol Start
1 - Calibrate Probes
2 - Equilibrate
3 - Loop 4 times
4 - (1-2-3-4)Mix for 3 Min. 0 sec.
5 - Time Delay of 0 Min. 0 sec.
6 - (1-2-3-4)Measure for 3 Min. 0 sec.
7 - Loop End
Program End
ABORT

12. When prompted, replace the calibration (utility) plate with the cell plate
13. Click OK, then Continue
14. The Optimization Assay will now run on the XF Analyzer
15. When the run is over, follow the prompts in the software and remove the cartridge and cell plate and discard


Experiment is Running
Protocol Start
1 - Calibrate Probes
2 - Equilibrate
3 - Loop 4 times
7 - Loop End
Program End
The calibration is now completed. Press OK when you are ready to load the optimization plate.
ABORT

Data Analysis and Interpretation

The XF Glycolysis Stress Test software output automatically graphs and analyzes the data from the optimization run, and offers a recommendation as to the cell seeding density required to achieve a minimum level of ECAR.

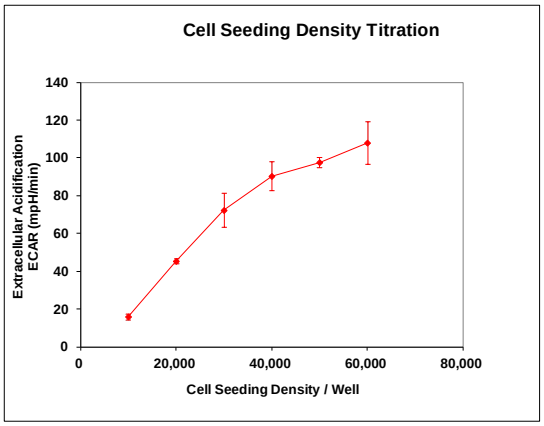


Figure 4. Cell Number Titration Optimization Graph

In Figure 4 cells were seeded at intervals of 10,000 cells/well. In this case, the seeding density recommended by the software is 20,000 cells/well.

While a particular seeding density may meet the technical requirements of the assay, these requirements should be balanced against biological factors. Observe the cell plate under a microscope before and after the experiment to assess both seeding uniformity and cell morphology. Cells which are overly sparse or crowded in the well may exhibit altered metabolic behavior, producing a higher degree of variation between wells.

The optimal cell seeding density should be determined based on the following criteria:

1. Minimal ECAR of 20 mpH/min
2. Seeding density falls within the linear portion of the graph

Visual confirmation of appropriate cell morphology and seeding.

NOTE: As the actual number of cells present in the well will affect the rate of glycolysis observed, it is strongly recommended to maintain a consistent number of hours between cell seeding and running the XF assay, to minimize variation between experiments.

Oligomycin Titration

The optimal concentration of Oligomycin can vary significantly between different cell types, culturing conditions, and experimental treatments. Therefore, the optimal Oligomycin concentration should be determined on any new cell type before running the XF Glycolysis Stress Test.

The Oligomycin Titration should be performed using the optimal cell seeding density, as determined in the Cell Number Titration experiment on page 9.

Prior to the Day of Assay

Cell Seeding

Seed cells ~24 hours prior to the assay in an XF cell culture microplate, and place in a 37°C incubator with the desired level of CO_2.

NOTE: As the actual number of cells present in the well will affect the rate of glycolysis observed, it is strongly recommended to maintain a consistent number of hours between cell seeding and running the XF assay to reduce variation between experiments. For experiments on non-adherent cells, seeding can be performed immediately prior to the experiment. Contact Seahorse Bioscience Support for more details.

Hydrating the XF Sensor Cartridge

The XF Sensor Cartridge must be hydrated prior to the assay:

- Add 1.0 mL of Seahorse Bioscience Calibrant to each well of an XF Utility Plate.
- Place the XF Sensor Cartridge on top of the utility plate, and place in a 37°C incubator **without** CO_2 for a minimum of 12 hours.

Day of Assay

Preparation of XF Glycolysis Stress Test Optimization Medium

Prepare the XF Glycolysis Stress Test Optimization Medium from the Base Medium, as described on page 8.

Perform the Medium Exchange

NOTE: Medium exchange described here is designed to produce a > 2,000 fold dilution of the original culture medium

1. Remove all but 50 µl of the culture medium from each well.
2. Rinse cells two times with 600 µl of Glycolysis Optimization Medium, pre-warmed to 37°C.
3. Add 670 µl to each well for a final volume of 720 µl/well
4. Place the plate in a 37°C incubator **without** CO_2 for one hour prior to the assay

NOTE: The Seahorse XF Prep Station is recommended to ensure accurate final volumes during media exchanges.

Prepare Oligomycin Titrations for Injection

Allow vial of DMSO and one aliquot of Oligomycin (5 mM) to thaw at room temperature. Label and refreeze the Oligomycin aliquot within 1 hour of thaw for subsequent experiments (do not discard). More than one additional freeze/thaw cycle is not recommended.

Create a serial dilution of Oligomycin in microtubes or suitable dilution vessel according to Figure 5.

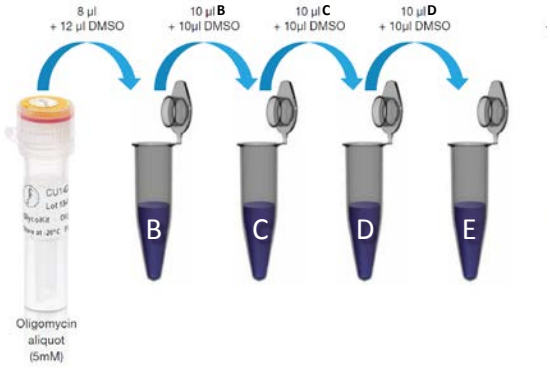
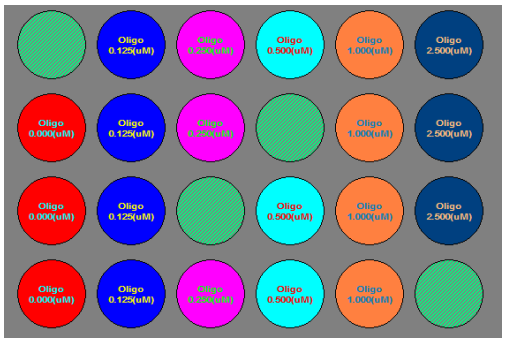


Figure 5. Oligomycin Serial Dilution Series

Add 7.5 µl of each dilution to 1493 µl of XF Glycolysis Stress Test Optimization Medium. Add 80 µl of the resulting solution to injection port A of the appropriate wells, following the plate layout shown at right.

NOTE: Injection ports of background wells should be loaded with the highest concentration of Oligomycin; however, no cells should be seeded in these wells.



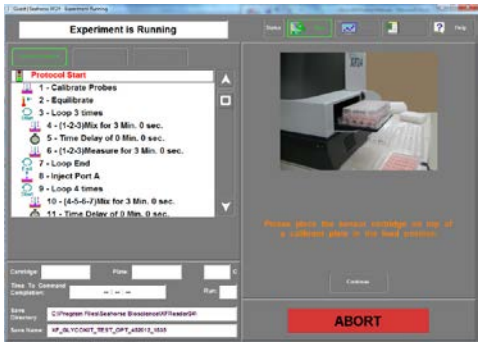
Once the injection ports are loaded, gently place cartridge in a CO_2-free incubator at 37°C until ready to begin the assay.

Running the Assay with the XF Glycolysis Stress Test Software App

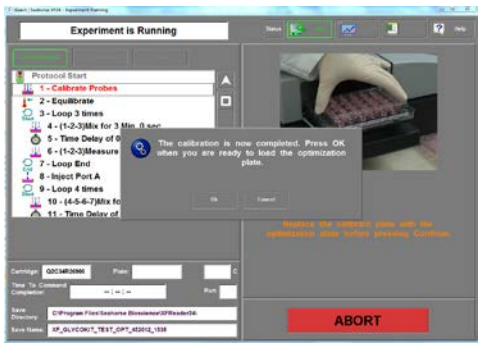
1. Open the XF software
2. In the Seahorse Apps drop-down menu, choose XF Glycolysis Kit
3. Click the Start App button
4. Click the Run Optimization Plate button
5. Select Oligo Titration from the drop down menu
6. Enter the Cell Line Name in the appropriate field
7. Enter the cell seeding number in the appropriate field

NOTE: If desired, the range of Oligomycin concentrations may be adjusted. In this case, the serial dilution volumes must also be adjusted accordingly.

8. Click the Start button
9. Choose a directory to save the file in, choose a file name, if necessary, and click OK
10. Place the cartridge and calibration plate with loaded injection ports on the sliding tray
11. Click Continue to start calibration



12. When prompted, replace the calibration (utility) plate with the cell plate
13. Click Ok, then Continue
14. The Optimization Assay will now run on the XF Analyzer
15. When the run is over, follow the prompts in the software and remove the cartridge and cell plate and discard


Experiment is Running
Protocol Start
1 - Calibrate Probes
2 - Equilibrate
3 - Loop 3 times
7 - Loop End
8 - Inject Port A
9 - Loop 4 times
The calibration is now completed. Press OK when you are ready to load the optimization plate.
ABORT

Data Analysis and Interpretation

The XF Glycolysis Stress Test software output automatically graphs and analyzes the data from the optimization run, and offers a recommendation as to the optimal Oligomycin concentration to use in the XF Glycolysis Stress Test.

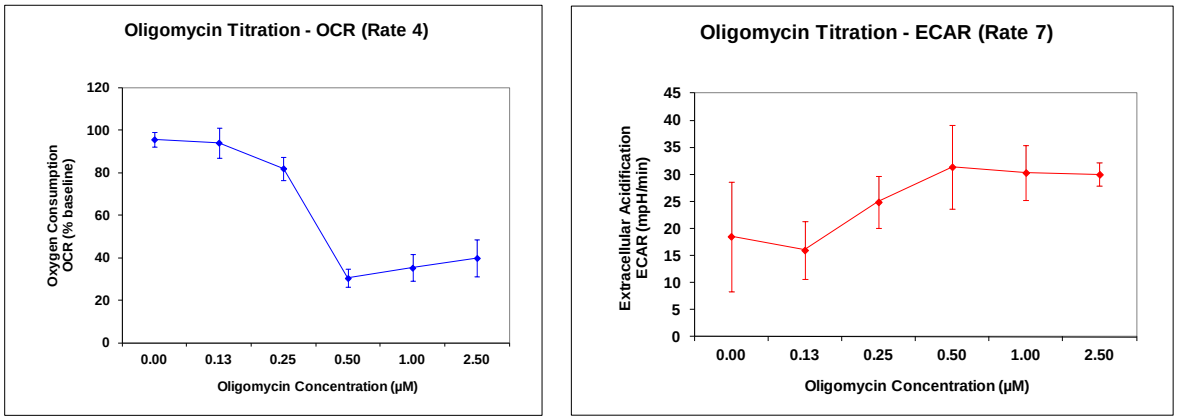


Figure 6: Oligomycin Titration Optimization Result Graphs

In Figure 6 the XF Glycolysis Stress Test software would recommend a concentration of 0.5 µM.

The optimal Oligomycin concentration should be determined based on the following criteria:

1. Maximum inhibition of OCR (as shown by the plateau in the dose response curve)
2. Maximum ECAR $\leq$ 120 mpH/min

If the maximum ECAR value exceeds 120 mpH/min, a lower cell seeding density should be selected, and the Oligomycin Titration should be repeated at this cell seeding density.

NOTE: As the actual number of cells present in the well will affect the rate of glycolysis observed, it is strongly recommended to maintain a consistent number of hours between cell seeding and running the XF assay to minimize variation between experiments.

IV. Running the XF Glycolysis Stress Test

Prior to the Day of Assay

Cell Seeding

Seed cells ~24 hours prior to the assay in an XF cell culture microplate, and place in a 37^{o} C incubator with the desired level of CO_2.

NOTE: As the actual number of cells present in the well will affect the rate of glycolysis observed, it is strongly recommended to maintain a consistent number of hours between cell seeding and running the XF assay to reduce variation between experiments.

NOTE: For experiments on non-adherent cells, seeding can be performed immediately prior to the experiment. Contact Seahorse Bioscience Support for more details.

Hydrating the XF Sensor Cartridge

The XF Sensor Cartridge must be hydrated prior to the assay:

- Add 1.0 mL of Seahorse Bioscience Calibrant to each well of an XF Utility Plate.
- Place the XF Sensor Cartridge on top of the utility plate, and place in a 37^{o} C incubator **without** CO_2 for a minimum of 12 hours.

Day of Assay

Preparation of XF Glycolysis Stress Test Assay Medium

Prepare the XF Glycolysis Stress Test Assay Medium from the Base Medium, as described on page 7.

Prepare Compounds for Injection

Thaw DMSO and one aliquot each of glucose (2.5 M), Oligomycin (5mM), and 2-DG (1 M) at room temperature.

1. Add glucose (1 mL) to 29 mL of XF Glycolysis Stress Test Assay Medium, pre-warmed to 37^{o}C. Adjust pH to 7.35 +/- 0.05.
    a. Final concentration of glucose in the assay will be 10 mM

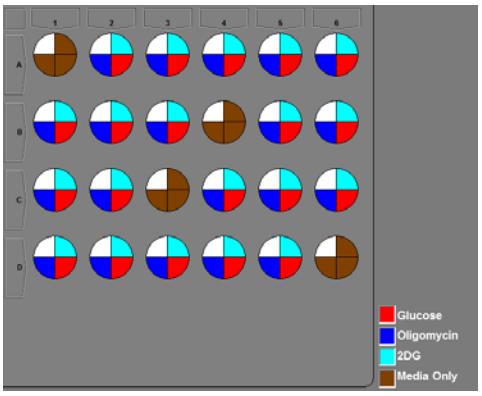
2. Prepare 3 mL of a 9x Oligomycin solution, based on the results of the titration performed in Section III - Optimization Assays – on page 9. See table below for easy reference.

Oligomycin Dose in the Assay	9x Solution for Injection	Volume of Oligomycin Stock	Volume of XF Glycolysis Stress Test Assay Medium
2.5 µM	22.5 µM	13.5 µl	2987 µl
1 µM	9 µM	5.4 µl	2995 µl
0.5 µM	4.5 µM	2.7 µl	2997 µl
0.25 µM	2.25 µM	1.35 µl	2999 µl
0.125 µM	1.125 µM	0.675 µl	2999 µl

3. Thawed 2-DG solution is ready to use
    a. Final concentration of 2-DG in the assay will be 100 mM

Load Injection Ports

Add 80 µl of the prepared compounds into the appropriate injection port, and place cartridge in a 37 °C incubator without CO_2:



Port A – Glucose
Port B – Oligomycin
Port C – 2-DG

Perform the Medium Exchange

NOTE: Medium exchange described here is designed to produce a > 2,000 fold dilution of the original culture medium

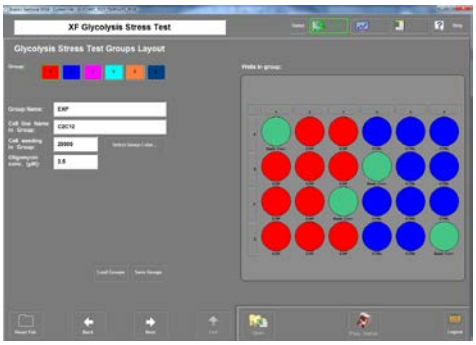
1. Remove all but 50 µl of the culture medium from each well.
2. Rinse cells two times with 600 µl of XF Stress Test Glycolysis Assay Medium, pre-warmed to 37^{o}C.
3. Add 510 µl to each well for a final volume of 560 µl/well
4. Place the plate in a 37°C incubator **without** CO_2 for one hour prior to the assay

NOTE: Using the Seahorse XF Prep Station is recommended to ensure accurate final volumes during media exchanges.

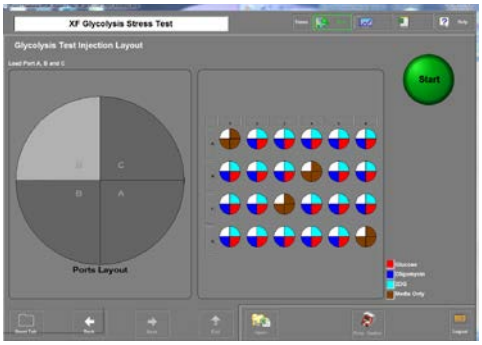
Running the Assay with the XF Glycolysis Stress Test Software App

1. Open the XF software
2. In the Seahorse Apps drop-down menu, choose XF Glycolysis Kit

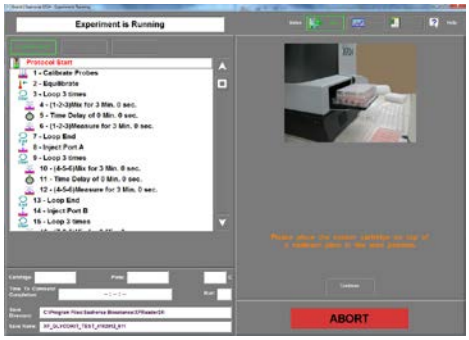
3. Click the Start App button
4. Click the Run XF Glycolysis Stress Test button
5. Enter the information for each group in the appropriate fields, and create the plate layout. When finished, click Next.



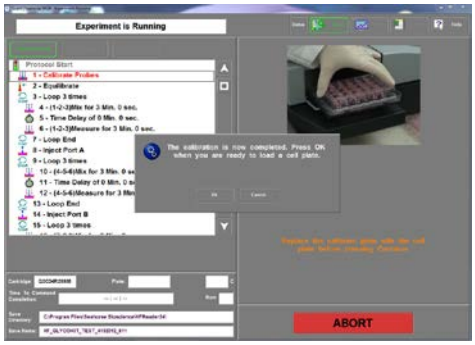
6. The injection port layout and details are show for reference. Click Start button to begin the assay.



7. Indicate the cell seeding density for each column by clicking on any well in the column, and entering the seeding density used
8. Choose a directory to save the file in, choose a file name, if necessary, and click OK
9. Place the cartridge and calibration plate with loaded injection ports on the sliding tray
10. Click Continue to start calibration



11. When prompted, replace the calibration (utility) plate with the cell plate
12. Click Ok, then Continue
13. The XF Glycolysis Stress Test will now run on the XF Analyzer
14. When the run is over, follow the prompts in the software and remove the cartridge and cell plate and discard



V. Analyzing the XF Glycolysis Stress Test Data

The XF Glycolysis Stress Test Software App delivers a series of automatic calculations, including the key parameters of glycolytic function – Glycolysis, Glycolytic Capacity, and Glycolytic Reserve.

Upon completion of the XF Glycolysis Stress Test, a tab is present entitled “Glycolysis Stress Test Results”, in addition to the standard data outputs from an XF Assay. Click on this tab to view the calculated metrics from the experiment.

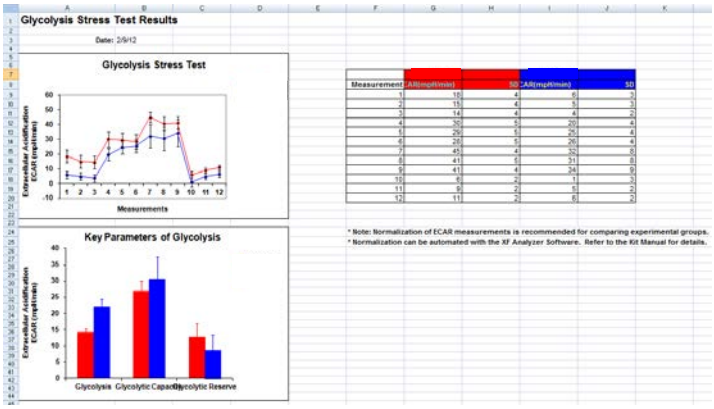


Figure 7: XF Glycolysis Stress Test Summary Results

Kinetic Results

This graph (top left) shows the average ECAR and standard deviation data obtained at each measurement point for all groups, in a graphical form. This data presentation highlights the dynamic changes within and between groups during the experiment.

Data Table

The numerical results from the kinetic graph are presented in tabular form to simplify data conversion/export for additional analyses.

Histogram

This graph (bottom left) shows the average ECAR and standard deviation data for the three key parameters of Glycolysis for all groups. In addition to these summary outputs, the XF Glycolysis Stress Test Software App also produces tabular and graphical presentations of the three key parameters of Glycolysis individually.

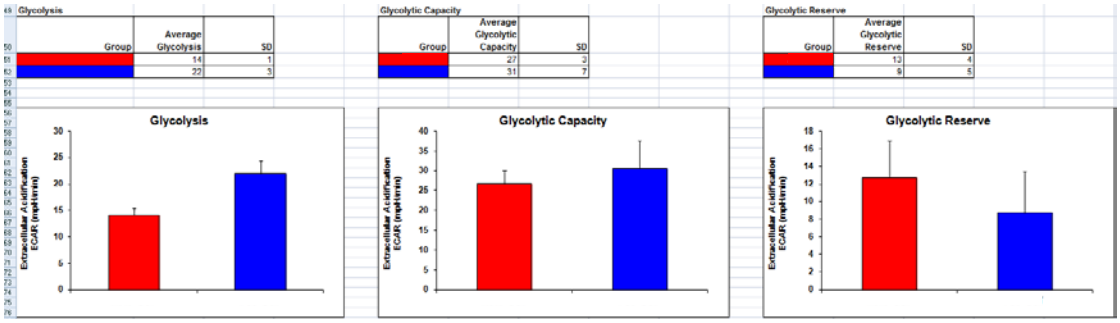


Figure 9: XF Glycolysis Stress Test Results Individual Parameters

NOTE: If changes are made in the Data Viewer tab, the assay must be re-exported using the XF Glycolysis Stress Test Software App to obtain the recalculated data output.

Reference:
Wu M, Neilson A, Swift AL, Moran R, Tamagnine J, Parslow D, Armistead S, Lemire K, Orrell J, Teich J, Chomicz S, Ferrick DA. Am J Physiol Cell Physiol; 2007 Jan 1. 292 (1):C125-136